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The aim of this project is to understand the mechanism of transition of breast cancer from hormone-dependent state to hormone-independent state. In order to pursue this question we have designed a regulatable regulator that can recognize all estrogen receptor target genes and put them under the regulation of exogenous compound RU486. In the presence of RU486, this regulator can bind to all estrogen targets and shut down their expression. We want to use this regulator to study the role of estrogen receptor target genes on breast cancer cell growth in hormone-dependent and hormone-independent states. In the last year, using a cellular culture system, we have successfully constructed this regulator and demonstrated that it can shut down a estrogen receptor reporter gene. With the success of this regulator, now we can answer what we set out to do in the next grant period.

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Introduction

Breast cancer is the most frequent cancer in American women (1). Despite extensive studies undertaken to understand the etiology of breast cancer, no clear picture has emerged. It is thought that genetic, dietary, hormonal, environmental and lifestyle factors contribute to the incidence of this disease (1). In contrast to many other cancers, the incidence of breast cancer has steadily increased with a 24% increase between 1973 and 1987 (1). Thus, breast cancer remains a major problem to overcome in the improvement of women's health in America.

Previous attempts to identify genes responsible for breast cancer have identified several oncogenes, such as wnt-1 (int-1) , wnt-3 and int-2, whose increased expression is frequently observed in MMTV-induced tumors(2). Furthermore, expression of these genes in mammary epithelial cells enables them to continue to grow even in a dense culture (3-5). These results provide strong evidence for their involvement in tumor development. The oncogenic potential of these genes has been demonstrated in transgenic animals. In addition, mutation of several tumor suppressor genes has also been shown to correlate with breast cancer (6,7).

Steroid hormones, estrogen and progesterone, and their receptors have also been demonstrated to be associated with breast cancer. Breast cell proliferated in response to estrogen and progesterone increases this proliferation potential. In addition, breast tumors rarely develop in ovariectomized woman (8). Furthermore, some women with breast cancer have higher estrogen levels than healthy control women (1) and antiestrogen treatment in breast cancer patients drastically reduces tumor reoccurrence (8,9). Finally, a strong correlation exists between reproductive history and the incidence of breast cancer (10).

It is well documented that initial breast cell growth and breast carcinoma is hormone-dependent. Antiestrogen treatment results in the arrest or remission of breast cancer growth (8,9). However, subsequently, most advance breast cancers become resistant to estrogen-ablation therapy (11). It has been proposed that mutation of the estrogen receptor (ER) to a constitutively active regulator or to a receptor which can be activated by estrogen antagonist, tomaxifen, may contribute to the transition from estrogen-dependent to -independent tumor growth [for review see (11)]. However, half of all advanced breast cancers are receptor positive but resistant to antiestrogen therapy and some ER negative tumors behave as if they are ER positive in expression of ER target genes such as progesterone receptor. Furthermore, many of ER mutations identified in tumor cells are also found in healthy cells of breast cancer patients or healthy individuals. thus, it remains controversial whether ER mutations have primary role in the transition from estrogen-dependent to -independent state.

In this proposal, we design experiments to dissect the role of ER target genes in the growth of breast cancer and to understand how transition from estrogen-dependent to-independent cancer growth occurs. We expect that results obtained from these studies will help us to devise a way to control breast tumor growth.

Originally we proposed three aims in this grant. However, reviewers deemed them too ambitious and recommended that the transgenic work proposed in the third Specific Aim be deleted. Thus, we concentrated our effort on the remaining two Specific Aims, the construction and characterization of a repressor system. The repressor system is based on the Ru486 regulatable system we have devised earlier. We have successfully constructed several regulators that can shut down the expression of ER dependent target reporter in a test transfection system. In addition, we have successfully generated stable MCF7 cell lines constitutively expressing Ru486 dependent regulator. Unfortunately, we were not able to demonstrate efficient down regulation of transfected reporter or endogenous ER target genes. Therefore, further improvement of this system is needed before it can be used to answer our proposed question on the role of ER target genes in estrogen independent cell growth.

A. Experimental Methods

(i) Plasmid construction

KRAB domain and E2F1 DNA binding domain were amplified from pBXG1/Kid-1N and pCMV-E2F1 and ligated together by PCR. The resultant fragment was cut by Xba1 and EcoRI and inserted into MCS sites of pBS-KSII(+) to constructed pKS-KE. The truncated PR-LBD(-19)-KRAB fusion fragment was amplified from pCEP4/GLK by PCR. The PCR product was cut by DraI and EcoRI, terminal end fill-in by klenow, then blunt-end ligated into EcoRV site of pKS-KE. The resultant plasmid pKS-KEPE contains functional KRAB domain at both N- and C-terminals of chimeric construct. E2F1 BNA binding domain was cut out using BamHI and EcoRI and replaced by PCR amplified ER DNA-binding domain to generate regulatable repressor of ER target genes pKS-KEDPK. The plasmid pKS-KEDPK was then subcloned into pCMX expression vector and checked in frame and by sequence analysis and by *in vitro* transcription/translation. The primers used for PCR amplification (5' primer; 3' primer) are as follows:

KRAB: AAGCTTCTAGACTGCAGCTCGAGGCCACCATGGCTCCTGAGCAAAG;
CCGCTTCACGGGATCCTCTCCTTGCTG.

E2F1-DBD: GAGGATCCCGTCAAGCGGAGGCTGGAC;
CCGGAATTCGGAGATCTGAAAGTTCTC.

ER-DBD: CGCGGATCCTATGGAATCTGCCAAGGAG;
CGGAATTCAGACCCCACTTCACCCCTG.

PR-LBD &
KRAB fusion: CGCGGATCCTTTAAAAAGTTCAATAAAGTCAGAG;
CCGGAATTCTCATCCTTGCTGCAACAGGGAG.

(ii) Transfection

Hela cells were routinely maintained in Dulbeco's Modified Medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT). Cells were seeded 24 hours before transfection in 6-well tissue culture plates (2 x 10 5 cells per well) in phenol red-free DMEM contained 10% charcoal/dextran treated FCS. DNA was introduced into cells using lipofectin (Gibco, Gaithersburg, MD) following the technique instruction. Cells were transfected for 6 hours and then washed with phosphate buffer to remove the lipofectin. Cells were incubated for an additional 24 hours in phenol red-free medium containing 10% charcoal/dextran treated FCS with or without hormones, as indicated in the text. Cell extracts were prepared by adding 30μl lysis buffer (Promega, Madison, WI) and assayed for luciferase activities (Monolight 2010 Luminometer, Analytical Luminescence Laboratory, MI). All determinations were performed in quadruple in at least two independent experiments.

(iii) Establishment of stable cell lines

MCF-7 cells were transfected with pcDNA3-KEDPK using calcium phosphate precipitation. The transfected cells were selected in the presence of 300 μg neomycin per ml of culture medium. The established neomycin resistant lines were then used for transfection and for characterizing of endogenous estrogen dependent target genes.

B. Results and Discussion

The plasmid pCMX-KEDPK expressing chimeric repressor of ER was constructed which contained a KRAB repressor domain at both N- and C-terminals, an ER DNA-binding domain and a truncated progesterone receptor ligand-binding domain. The construct was confirmed by sequence analysis. In addition, we have constructed the plasmid pCMX-EDPK which has only one KRAB repressor domain at the C-termials. An expected size of the chimeric proteins were produced by *in vitro* transcription/translation (Figure 1). We also constructed two constructs with estrogen and progesterone receptor ligand binding domains that replace the mutated PR ligand binding domain as originally proposed. Since they are only being used in transgenic experiments, they were not pursued further. The capacity of repressor KEDPK to block the ER mediated transcription was tested by co-transfecting plasmids carrying KEDPK, human ER and 3(ERE) tataLuc into HeLa cells (Figure 2). The luciferase activity was measured 24 hours following treatment with 17β-estradiol and Ru486. In the presence of Ru486 (10nM) the repressor KEDPK significantly inhibits the luciferase activity induced by ER. Fifty percent inhibition was observed when transfected equally amount of ER and repressor KEDPK. The inhibitory potency of KEDPK on ER transcription activity was shown in a dose-dependent manner (Figure 3). KEDPK did not interfere the transcription activity of ER in the absence of Ru486. The inhibitory activity of KEDPK was tightly regulated by Ru486, with maximal effect at concentration of 10 nM (Figure 4). Similar results were observed in breast cancer cell line MCF-7. The inhibitory activity of KEDPK was specific to the ER, since the repressor has shown no effect on other nuclear receptor systems tested so far in transient transfection (Figure 5). In addition, we analyzed the effect of KDEPK repressor on the partial agonist activity of 4hydrxy-tamoxifen (4OH-T) by cotransfection of KDEPK and estrogen receptor expression vectors and reporter gene in the presence of 4OH-T. As shown in Figure 6, the expression of KDEPK inhibits both estrogne and 4OH-T dependent agonist activity of ER. Thus, KDEPK is effective in inhibiting reporter containing ER binding site. To establish that this inhibition is of physiological significance, we use a reporter gene with nature ER dependent promoter, complement 3 factor (C3). As shown in Figure 7, KEDPK is able to inhibit more than 70% of the promoter activity of C3.

It is concluded that the chimeric repressor we designed could potentially and specifically inhibit ER target gene expression in response to exogenous ligand. This provided a useful tool to study the role of ER target genes during breast cancer progression, and can be used as innovative strategies for gene therapy of breast cancer. In order to establish the usefulness of this construct in inhibiting estrogen target genes, we have established cell lines from E_2-dependent MCF7 and E_2-independent MCF7-derived LTSD cells.

The three established cell clones were then transfected with $(ERE)_3$ TATA Luc reporter to determine whether inserted KEDPK construct was able to be expressed enough repressor to inhibit transfected ERE reporter gene. As shown in Figure 8, the KEDPK is only able to inhibit partially the ER dependent activity. Thus,

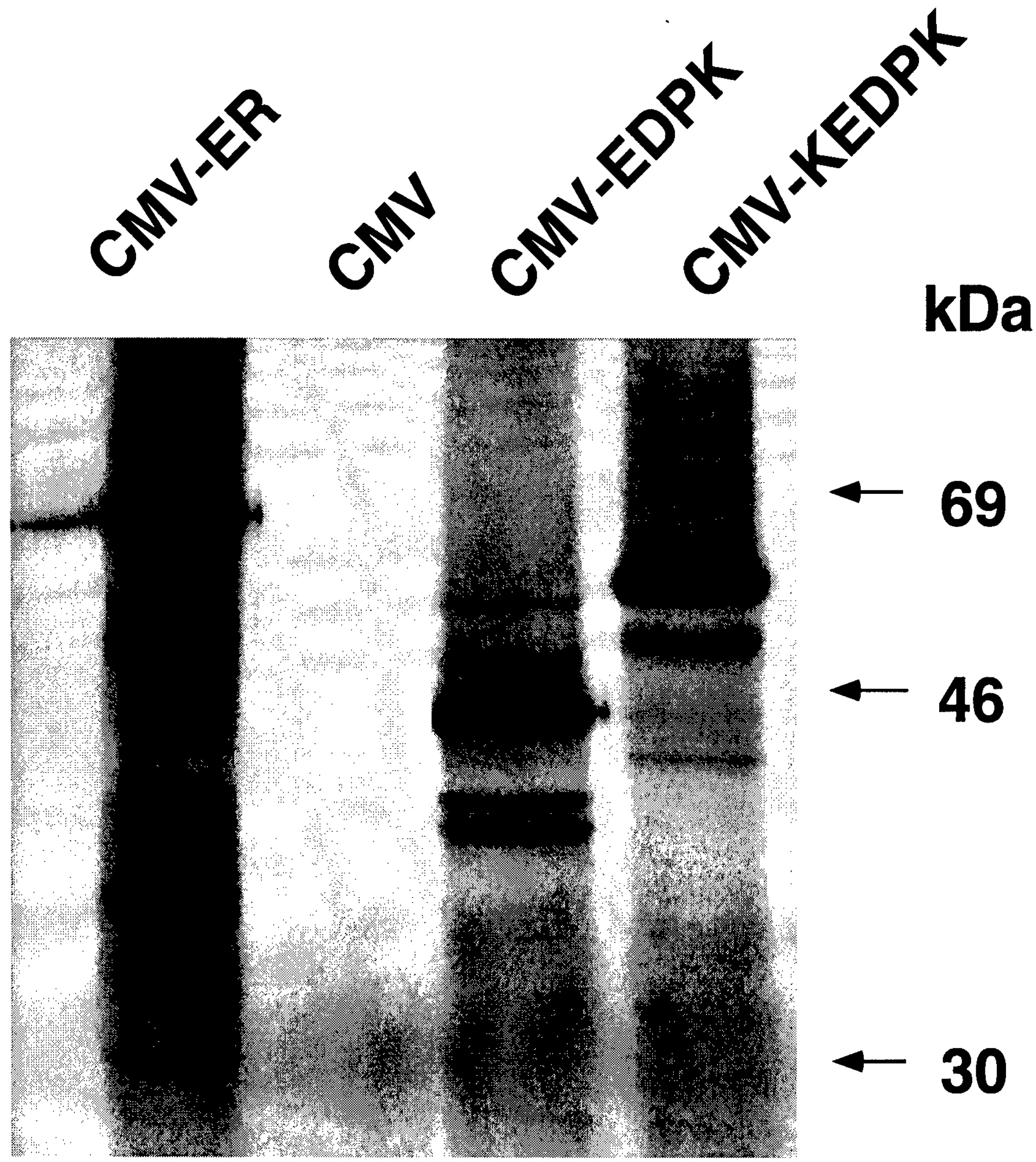

FIGURE 1. In Vitro Transcription/Translation of the Regulatable Repressor, KEDPK

A TNT coupled reticulocyte lysate systems was used to express KEDPK regulator. An empty expression vector and ER expression vector were included as control. The translation products were separated by 10% SDS-PAGE. The expected protein size of approximately 62 kDa was confirmed by the translation product of the KEDPK expression vector. No protein was translated by the empty vector. The data suggested that the recombinant DNA construct was able to express the chimera protein of repressor, KEDPK.

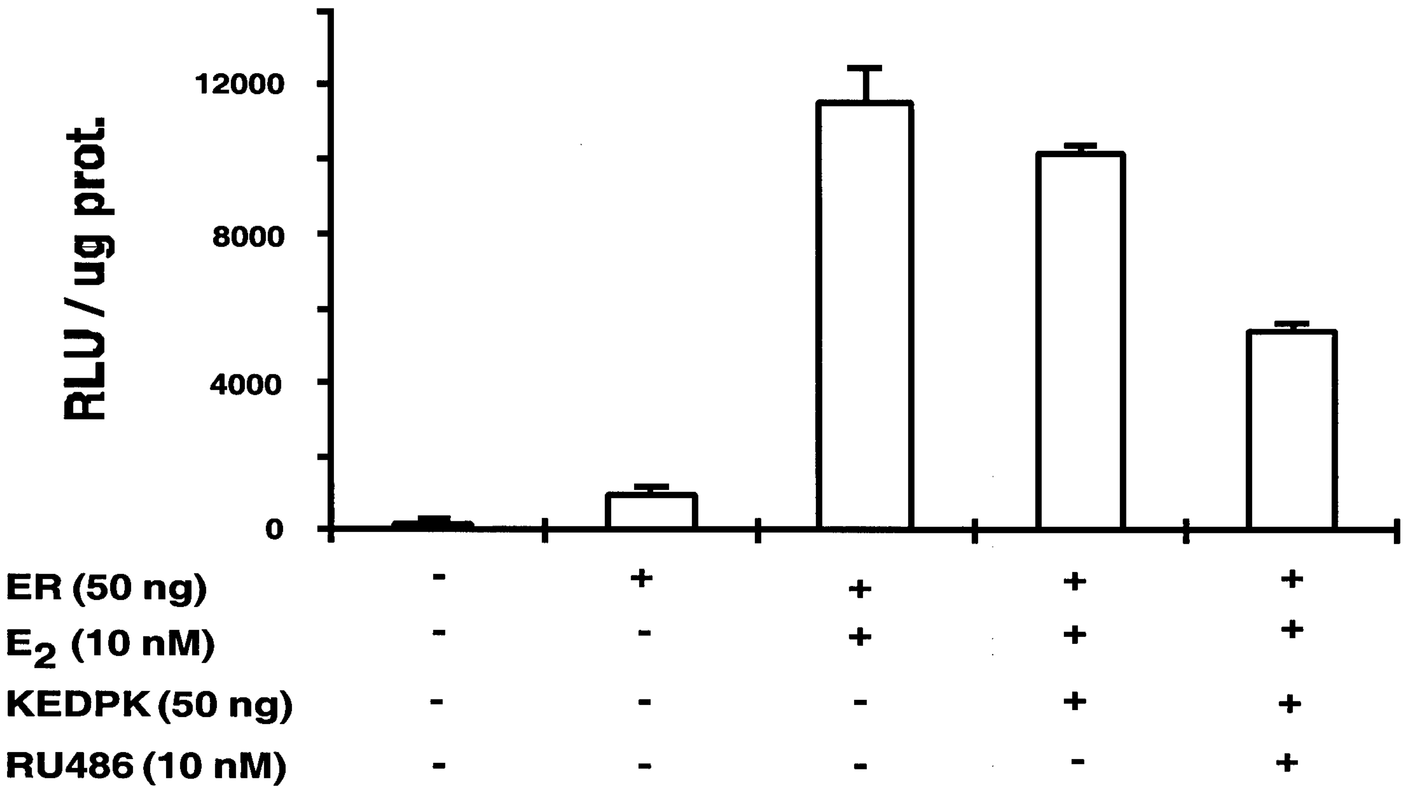

FIGURE 2. Inhibition of ER-dependent Activation by Regulatable Repressor, KEDPK

(ERE)3tataLuc reporter gene was transfected along with human ER expression plasmid and regulatable repressor, KEDPK, in Hela cells using lipofectin. After 6 hr of transfection, cells were washed and incubated for an additional 24 hr either with or without hormones, as indicated. Cell extracts were prepared and assayed for luciferase activities. The regulatable repressor KEDPK significantly inhibit the ER mediated transcription in the presence of RU486.

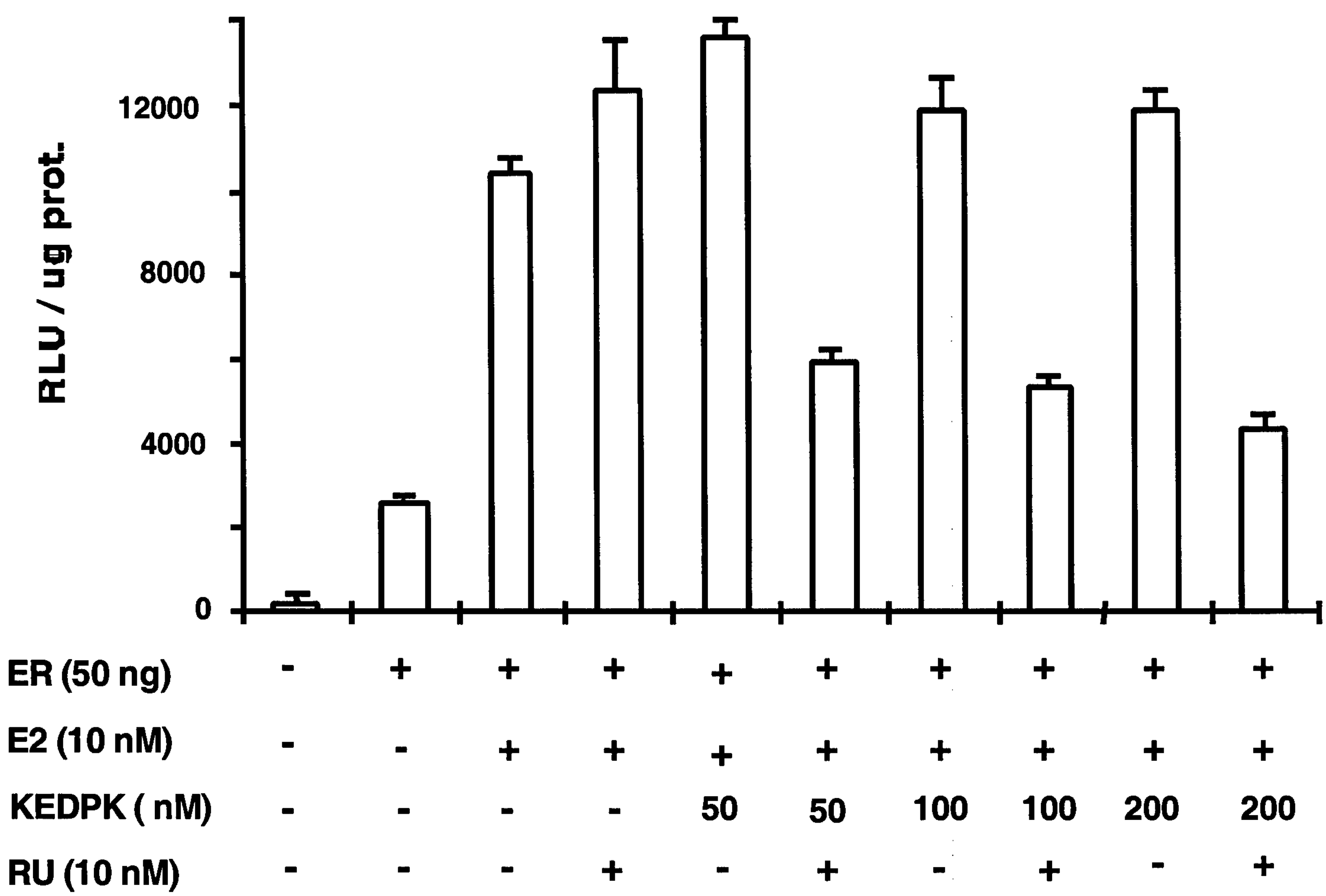

FIGURE 3. Inhibition of ER Activity by KEDPK in a Dose-Dependent Manner

Cells were transfected with (ERE) 3tataLuc reporter, human ER expression plasmid, and an indicated amount of KEDPK repressor construct. Cells were treated with 10 nM 17ß-estradiol and RU486 for 24 hrs as indicated. As shown, the luciferase activity induced by ER was inhibited in the presence of increasing amounts of KEDPK-expressing vector, indicating that the inhibitory effect of KEDPK was dose-dependent.

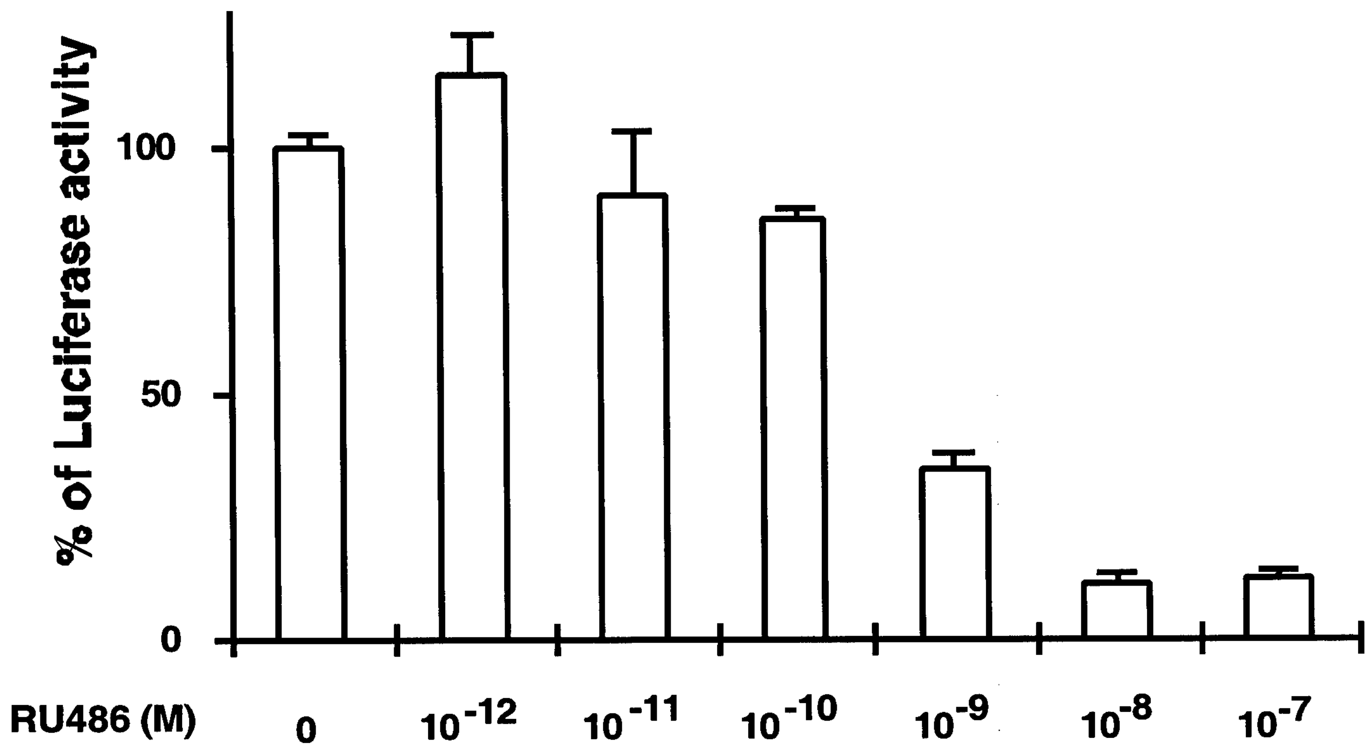

FIGURE 4. Induction of KEDPK Repression Activity by RU486 in a Dose-Dependent Manner

HeLa cells were transiently transfected with 50 ng (ERE)3tataLuc reporter, 50 ng human ER expression plasmid, and 200 ng repressor construct, KEDPK in the pressence of 17ß-estradiol (10 nM) and different concentrations of RU486 for 24 hrs. In the absence of RU486, KEDPK did not interfere with transcription activity. The inhibitory activity of KEDPK was observed only in the presence of RU486, with a maximal effect at the concentration of 10 nM.

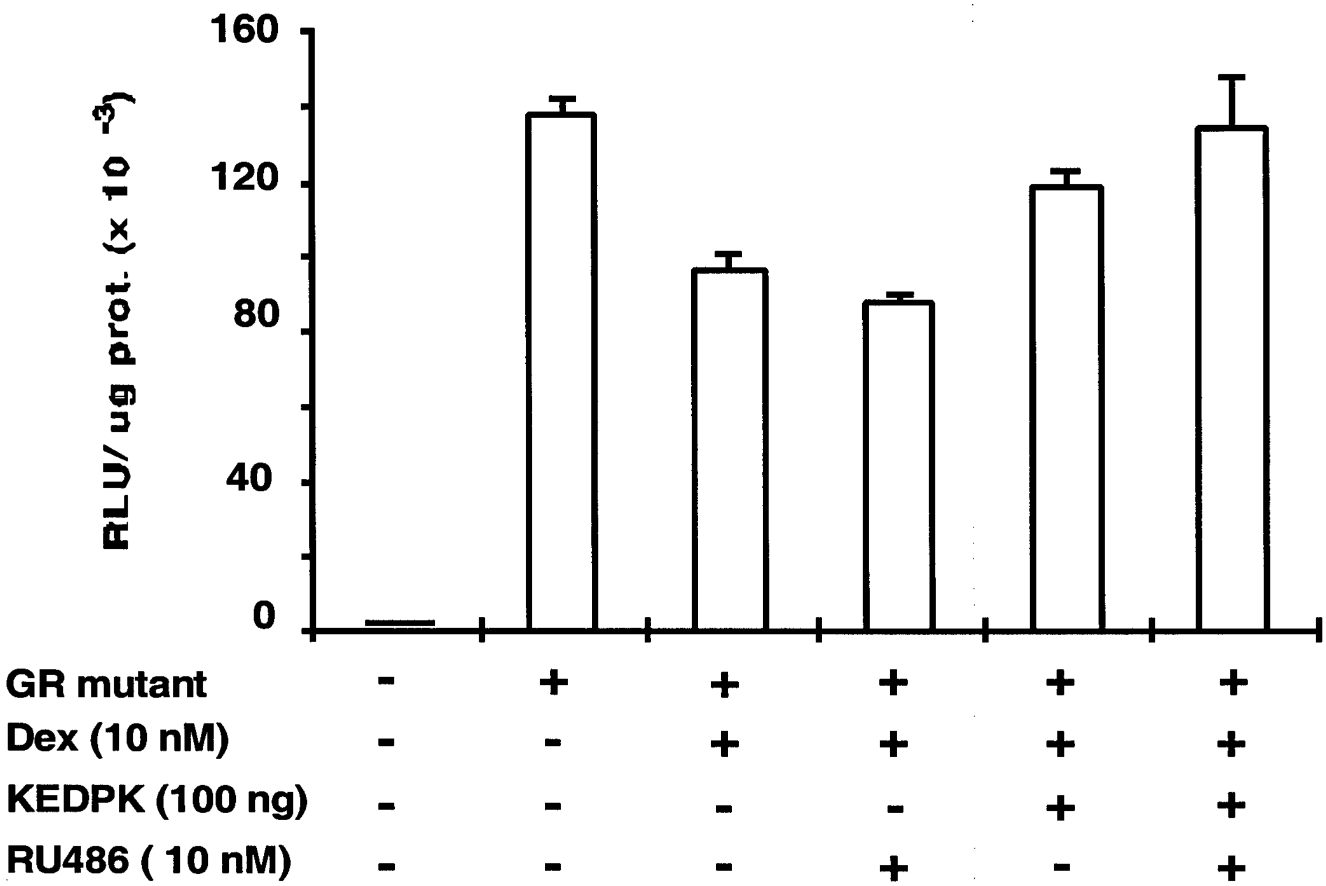

FIGURE 5. Effect of KEDPK on Glucocorticoid Receptor (GR) Mediated Transcriptional Activity

Cells were transfected with 50 ng of reporter GREtkLuc, 50 ng of GR mutant 3556 (LBD truncation), and 100 ng regulatable repressor, KEDPK. The GR mutant binds to GR response element (GRE) and constitutively activates the gene expression independent of its cognate ligand. The regulatable repressor KEDPK has no effect on the transcription induced by the GR mutant, either in the absence or presence of RU486. The results suggested that the inhibitory effect of KEDPK was specific to the ER target genes.

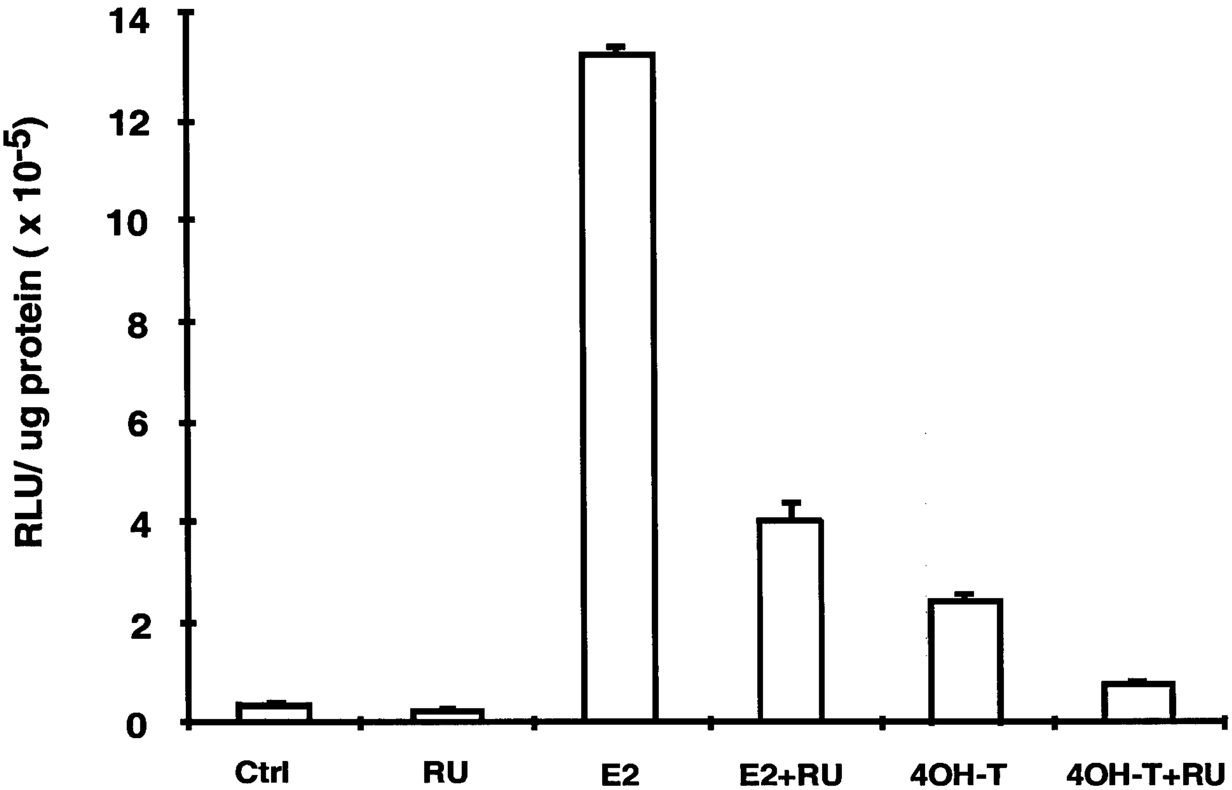

FIGURE 6. Effect of KDEPK on 4-Hydroxy-tamoxifen (4OH-T) Stimulated ER Activity

The repressor plasmid (200ng) and (ERE)3 tata Luc reporter (100 ng) were transfected together with ER expression vector (100ng) into HepG2 (human heptocellular carcinoma) cells where AF1 activity of ER was proven to be dominant (Tzukerman MT, 1996). 4-hydroxy-tamoxifen (4OH-T) treatment of HepG2 cells resulted in a eleven-fold induction of ER mediated transcription (10% of E2). Repressor, KEDPK, could inhibit the 4HOT-induced ER activity as efficiently as that on the E2 induced activity.

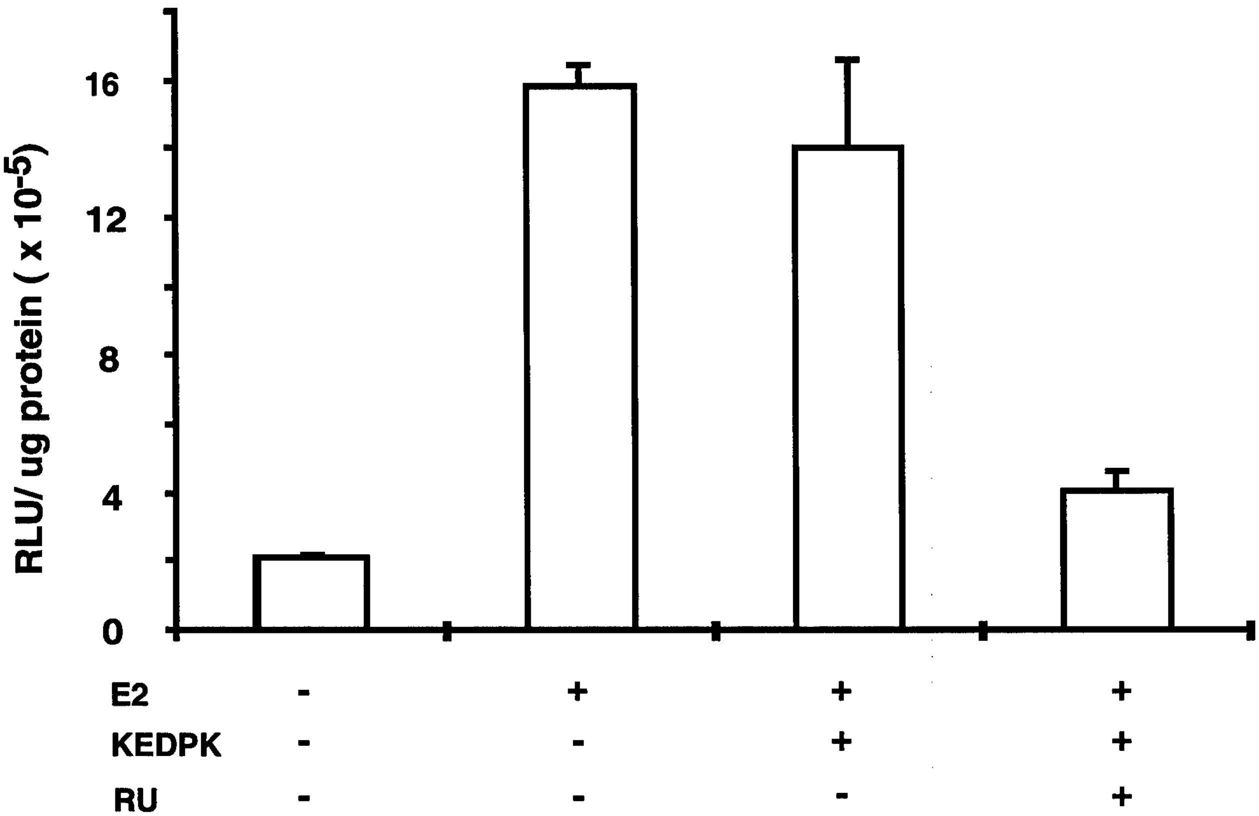

FIGURE 7. KEDPK Is Able to Inhibite the Promoter Activity of Complement Component 3 Factor (C3)

Hela cells were transfected with C3-Luc promoter which contains 1.8 kb of human C3 gene promoter (-1808 to 58), ER expression plasmid, and KEDPK repressor construct. Cells were treated with 10 nM 17ß-estradiol and RU486 for 24 hrs as indicated. E2 stimulated C3 transcription was almost completely blocked in cells transfected with repressor after treatment of RU486 suggesting that the repressor KEDPK appears to suppress more effectively the ER avtivity on endogenous promoter than on artificial (ERE)3 tata reporter.

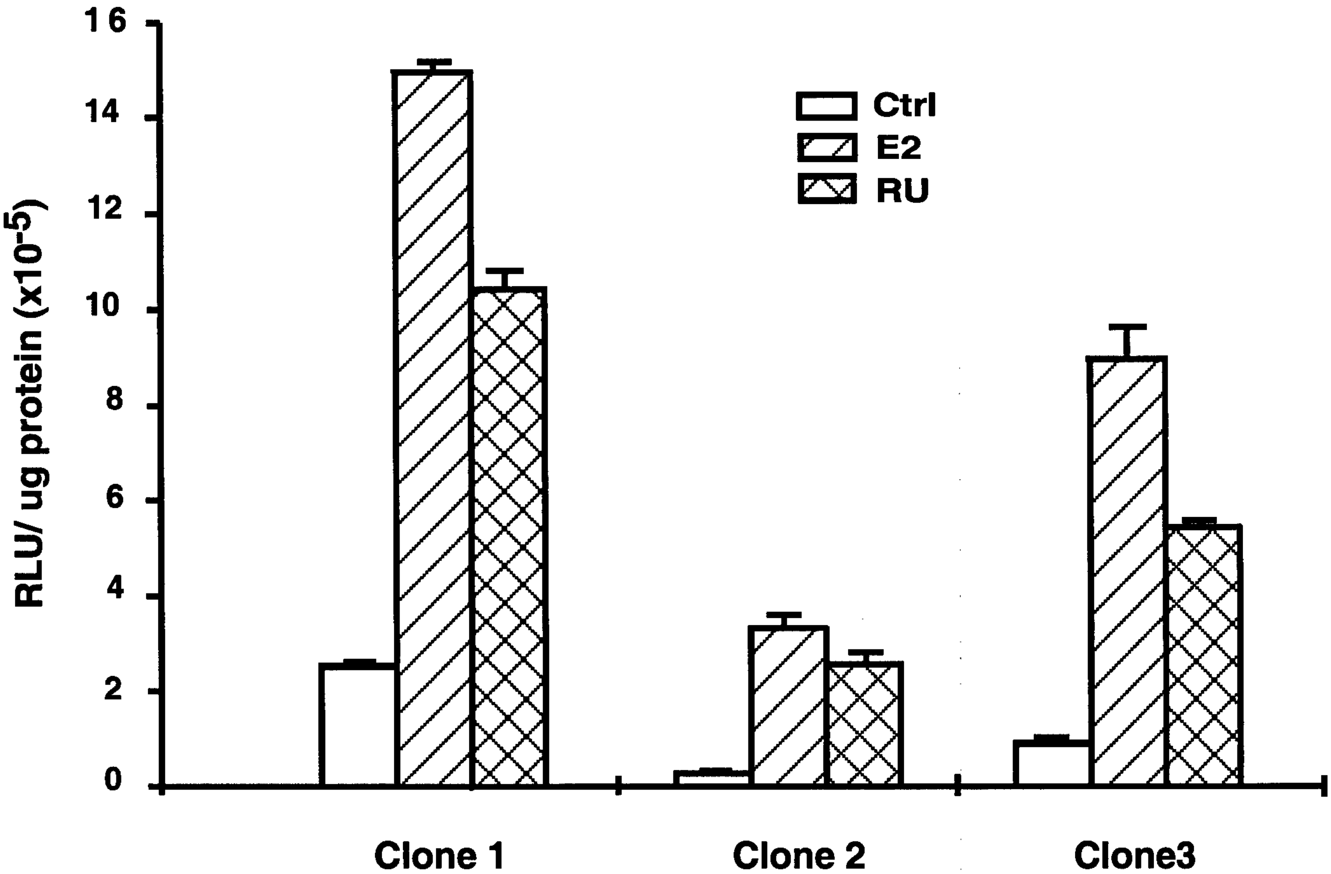

FIGURE 8. Functional Screening of MCF-7 Cells Stably Transfected with KEDPK

MCF-7 cells were transfected with pcDNA3-KEDPK using calcium phosphate precipitation. The transfected cells were selected in the presence of 300 μg neomycin per ml of medium. Neomycin-resistant cells were then taken and cultured seperately. 100 ng (ERE)3 tata-Luc reporter was transfected into the cells. Luciferase activity was determined 24 hrs after 17β-estradiol and RU486 treatment. Results indicate that selected clones respond differently to RU486 in suppression of the ER mediated transcription.

stable integrated KEDPK is not expressed high enough levels to effectively compete with the endogenous ER in MCF7 cells. Similarly, when we measure the endogenous ER Target gene PS2 and progesterone, we found very little inhibition can be observed (data not shown). Although this observation is disappointing, it indicates that we should be able to reach our goal if we can obtain a cell clone which expresses high leves of KEDPK. We currently continue to select stable cell lines with the hope that we will be able to obtain a high expressor for future studies.

Conclusions

In the last two years, we have made major progress toward our goals to understand the transition of breast cancer cells from E2-dependent to E2-independent state. We have successfully constructed a regulator that can shut down ER target genes in a Ru486-dependent manner. We have tested it in a transfection system and it was shown to work well and specifically. We also successfully established stable cell lines from E2-dependent and -independent MCF7 cells. Its validity and effectiveness in controlling breast cancer cell growth will be tested in the coming year. We are optimistic that we will accomplish what we have proposed to do.

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